Hypoxia positively regulates the expression of pH-sensing G-protein-coupled receptor OGR1 (GPR68)

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Abstract: BACKGROUND AIMS A novel family of proton-sensing G-protein-coupled receptors, including ovarian cancer G-protein-coupled receptor 1 (OGR1) (GPR68) has been identified to play a role in pH homeostasis. Hypoxia is known to change tissue pH as a result of anaerobic glucose metabolism through the stabilization of hypoxia-inducible factor-1 . We investigated how hypoxia regulates the expression of OGR1 in the intestinal mucosa and associated cells. METHODS OGR1 expression in murine tumors, human colonic tissue, and myeloid cells was determined by quantitative reverse-transcription polymerase chain reaction. The influence of hypoxia on OGR1 expression was studied in monocytes/macrophages and intestinal mucosa of inflammatory bowel disease (IBD) patients. Changes in OGR1 expression in Mono-Mac6 (MM6) cells under hypoxia were determined upon stimulation with tumor necrosis factor (TNF), in the presence or absence of nuclear factor- B (NF- B) inhibitors. To study the molecular mechanisms involved, chromatin immunoprecipitation analysis of the OGR1 promoter was performed. RESULTS OGR1 expression was significantly higher in tumor tissue compared with normal murine colon tissue. Hypoxia positively regulated the expression of OGR1 in MM6 cells, mouse peritoneal macrophages, primary human intestinal macrophages, and colonic tissue from IBD patients. In MM6 cells, hypoxia-enhanced TNF-induced OGR1 expression was reversed by inhibition of NF- B. In addition to the effect of TNF and hypoxia, OGR1 expression was increased further at low pH. Chromatin immunoprecipitation analysis showed that HIF-1 , but not NF- B, binds to the promoter of OGR1 under hypoxia. CONCLUSIONS The enhancement of TNF- and hypoxia-induced OGR1 expression under low pH points to a positive feed-forward regulation of OGR1 activity in acidic conditions, and supports a role for OGR1 in the pathogenesis of IBD.

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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Hypoxia Positively Regulates the Expression of pH-Sensing G-Protein–Coupled Receptor OGR1 (GPR68)

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SUMMARY

Hypoxia induces the expression of pH-sensing ovarian cancer G-protein–coupled receptor 1 (GPR68) in monocytes/macrophages and intestinal mucosa of inflammatory bowel disease patients, which is enhanced further at acidic pH in monocytes/macrophages. Hypoxia-inducible factor-1α binds to the ovarian cancer G-protein–coupled receptor 1 promoter under hypoxia, pointing to the direct regulation of this gene.

BACKGROUND & AIMS: A novel family of proton-sensing G-protein–coupled receptors, including ovarian cancer G-protein–coupled receptor 1 (OGR1) (GPR68) has been identified to play a role in pH homeostasis. Hypoxia is known to change tissue pH as a result of anaerobic glucose metabolism through the stabilization of hypoxia-inducible factor-1α. We investigated how hypoxia regulates the expression of OGR1 in the intestinal mucosa and associated cells.

METHODS: OGR1 expression in murine tumors, human colonic tissue, and myeloid cells was determined by quantitative reverse-transcription polymerase chain reaction. The influence of hypoxia on OGR1 expression was studied in monocytes/macrophages and intestinal mucosa of inflammatory bowel disease (IBD) patients. Changes in OGR1 expression in MonoMac6 (MM6) cells under hypoxia were determined upon stimulation with tumor necrosis factor (TNF), in the presence or absence of nuclear factor-κB (NF-κB) inhibitors. To study the molecular mechanisms involved, chromatin immunoprecipitation analysis of the OGR1 promoter was performed.

RESULTS: OGR1 expression was significantly higher in tumor tissue compared with normal murine colon tissue. Hypoxia positively regulated the expression of OGR1 in MM6 cells, mouse peritoneal macrophages, primary human intestinal macrophages, and colonic tissue from IBD patients. In MM6 cells, hypoxia-enhanced TNF-induced OGR1 expression was reversed by inhibition of NF-κB. In addition to the effect of TNF and hypoxia, OGR1 expression was increased further at low pH. Chromatin immunoprecipitation analysis showed that HIF-1α, but not NF-κB, binds to the promoter of OGR1 under hypoxia.

CONCLUSIONS: The enhancement of TNF- and hypoxia-induced OGR1 expression under low pH points to a positive feed-forward regulation of OGR1 activity in acidic conditions, and supports a role for OGR1 in the pathogenesis of IBD. *(Cell Mol Gastroenterol Hepatol 2016;2:796–810; http://dx.doi.org/10.1016/j.jcmgh.2016.06.003)*

Keywords: Ovarian Cancer G-Protein–Coupled Receptor; Inflammation; Inflammatory Bowel Disease; TDAG8; GRP65.

Cells in diseased tissues, such as malignant tumors, atherosclerotic plaques, arthritic joints, and chronically inflamed tissue, experience prolonged periods of hypoxia. A family of hypoxia-inducible factor (HIF) transcription factors is predominately responsible for mediating cellular adaptation to low oxygen availability.[1,2] HIF-1α has been implicated in a number of inflammatory diseases including rheumatoid arthritis, allergic asthma, psoriasis, and inflammatory bowel disease (IBD).[3]

Hypoxia and inflammation are interconnected and linked on many levels and may induce and influence each other in various ways.[4] Inflammation may be hypoxia-driven or hypoxia may be induced by inflammation (inflammatory hypoxia).[4] Hypoxia not only maintains or aggravates inflammation via stabilization of HIF-1α, but it also

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Abbreviations used in this paper: **AICAR, 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside; CD, Crohn's disease; ChIP, chromatin immunoprecipitation; FCS, fetal calf serum; GPR, G-protein–coupled receptor; HIF, hypoxia-inducible factor; HV, healthy volunteer; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; MM6, MonoMac 6; mRNA, messenger RNA; NF-κB, nuclear factor-κB; OGR1, ovarian cancer G-protein–coupled receptor 1 (GPR68); RT-qPCR, quantitative reverse-transcription polymerase chain reaction; SPARC, secreted protein acidic and rich in cysteine; TDAG8, T-cell death-associated gene 8 (GPR65); Th, T-helper; TNF, tumor necrosis factor; UC, ulcerative colitis; WT, wild type.**

Most current article

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influences the local tissue pH in the mucosa.[5] Subsequently, an acidic environment is not only the result of an inflammation, but also affects the degree and outcome of inflammation.[6–8] Inflammation has been attributed to an increase in local proton concentration and lactate production,[9–11] and linked to subsequent proinflammatory cytokine production, such as tumor necrosis factor (TNF), interleukin (IL)6, interferon-γ, and IL1β.

Hypoxia is also a consequence of solid tumor formation. Larger colorectal cancers with insufficient angiogenesis are characterized by a lack of oxygen supply. However, even in the case of sufficient oxygen supply, as first described by Warburg et al[12] in 1924, cancer cells preferentially may metabolize glucose to lactate by an anaerobic pathway (known as the Warburg effect). Lactate induces the expression of HIF-1α and causes acidification of the surrounding tissue.[13-15]

Hypoxia inflicts a broad spectrum of effects on the cellular, organ, and systemic levels. In the intestine, hypoxic conditions affect different processes including absorption, metabolism, and inflammation.[16,17] During mucosal inflammation, edema, vasculitis, and vasoconstriction separate epithelial and other cells from the blood supply and thus access to oxygen. Karhausen et al[18] showed that even at basal conditions some extent of hypoxia is detectable in the superficial epithelial layers of the murine colon. After induction of colitis in a mouse model, aggravated hypoxia occurred, and even reached submucosal regions.[18]

HIF-1α is an oxygen-sensing transcription factor that regulates the expression of various genes enhancing oxygen delivery or promotes cell survival under hypoxic conditions. Heterodimeric transcription factor HIF-1α undergoes oxygen-dependent hydroxylation, which leads to binding of the von Hippel-Lindau tumor suppressor protein and subsequent ubiquitin-mediated proteosomal degradation. No hydroxylation occurs under hypoxic conditions, followed by an accumulation of HIF-1α and binding to HIF-1β, forming the active HIF-1 complex. The active HIF-1 dimer binds to hypoxia response elements in the DNA, thereby leading to the expression or suppression of its target genes.[19]

A family of G-protein–coupled receptors, which includes ovarian cancer G-protein–coupled receptor 1 (OGR1, also known as G-protein–coupled receptor [GPR]68), GPR4, and T-cell death-associated gene 8 (TDAG8, also known as GPR65), sense extracellular protons through imidazole groups on histidine residues located on the extracellular region of the receptor.[20,21] Signaling pathways induced by protons binding to the receptor include phospholipase C activation, inositol trisphosphate formation, and subsequent Ca^{2+} release via the G_q pathway[20,22,23] or cyclic adenosine monophosphate production through the G_s pathway.[24,25]

Recent studies have shown a link between IBD and this family of pH-sensing G-protein–coupled receptors. TDAG8 has been identified as an IBD risk gene by association results and meta-analysis of genome-wide association studies.[26,27] Furthermore, single-cell RNA sequencing and computational analysis, which is used to determine different cellular states of T-helper (Th)17 cells and rank genes based on their pathogenicity, recently has shown that TDAG8 promotes Th17 cell pathogenicity.[28] In addition, chromatin immunoprecipitation (ChIP) sequencing analysis shows that the Th17 cell master transcription factor (Rorγt) binds the promoter region of GPR65.[29]

In a previous study we reported that IBD patients expressed higher levels of OGR1 messenger RNA (mRNA) in the mucosa than healthy control subjects.[22] We also observed that OGR1 expression is induced in cells of human macrophage lineage and primary human monocytes by TNF, whereby this effect is reversed by inhibition of the key regulator of chronic mucosal inflammation, nuclear transcription factor-κB (NF-κB). These studies proposed a role for OGR1 in the development of mucosal inflammation, showing that accumulation of extracellular protons promotes predominately G_q signaling in monocytes/macrophages and intestinal epithelial cells (IECs), causing modulation of cellular responses, such as F-actin stress fiber formation and tightening of the epithelial barrier.[22,23]

Hypoxia induces a decrease in tissue pH that may be sensed by OGR1, thereby influencing inflammation, however, information on its signaling remains limited. Therefore, we investigated the role of OGR1 during hypoxia associated with inflammation. We studied the expression of OGR1 in a human monocyte model, in primary intestinal macrophages and in IBD patients subjected to hypoxia. We show that OGR1 expression is enhanced by hypoxia. In a human cell model subjected to hypoxia, TNF-induced OGR1 expression was abrogated by NF-κB inhibition.

Materials and Methods

Chemicals

All chemicals and cytokines were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. All cell culture reagents were obtained from Thermo Fisher (Allschwil, Switzerland), unless otherwise specified. TNF (#654205) was purchased from Calbiochem (Merck, Darmstadt, Germany). 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) (#100102-41), BAY-11-7082 (#100010266), CAY10512 (#10009536), curcumin (#81025.1), SC-514 (#10010267), and SP600125 (#100010466) were purchased from Cayman (Ann Arbor, MI). The custom monoclonal anti-OGR1 antibody was developed by AbMart (Shanghai, China).

Human Subjects and Exposure to Hypobaric Hypoxia

Human colon biopsy specimens were taken from patients with Crohn's disease (CD) or ulcerative colitis (UC), or from healthy volunteers (HVs). HVs (n = 10), CD patients (n = 11), and UC patients (n = 9) in clinical remission were subjected to hypoxic conditions in a hypobaric chamber resembling an altitude of 4000 m above sea level for 3 hours. Clinical activity in patients with CD and in patients with UC was assessed using the Harvey–Bradshaw Index and the Partial Mayo Score, respectively. The participant characteristics are shown in Table 1. Distal colon biopsy specimens were collected the day before entering the hypobaric chamber (T1), immediately after hypoxia (T2), and 1 week after the first biopsy (T3) at the Division of Gastroenterology and Hepatology of the University Hospital Zurich. Total RNA was isolated, reverse-transcribed,

Table 1. Participant Characteristics

Item	HVs	CD	UC
Number of patients	10	11	9
Sex: females, n	3 (30%)	6 (54.5%)	5 (55.6%)
Age, mean ± SD, y	28.2 ± 4.9	35.2 ± 13.3	31.8 ± 10.8
Smoking status	2/10 (20.0%)	3/11 (27.3%)	2/9 (22.2%)
Disease severity			
Harvey–Bradshaw Index, median (IQR)	NA	0.0	NA
Partial Mayo score, median (IQR)	NA	NA	0.0
Medical history			
Azathioprine/6-MP	NA	5/11 (45.5%)	7/9 (58.3%)
Methotrexate	NA	1/11 (9.1%)	0/9 (0.0%)
Anti-TNF	NA	6/11 (54.5%)	2/9 (16.7%)
Systemic steroids	NA	1/11 (9.1%)	3/9 (25.0%)
NSAID intake	NA	2/11 (18.2%)	6/9 (75.0%)

IQR, interquartile range; MP, mercaptopurine; NA, not applicable; NSAID, nonsteroidal anti-inflammatory drug.

and hypoxia-induced changes in gene expression were analyzed using quantitative reverse-transcription polymerase chain reaction (RT-qPCR). This study was approved by the Ethics Committee of the Canton of Zurich (KEK-ZH no. 2013-0284) and all participants signed an informed consent.

Human Intestinal Macrophage Isolation

Surgical specimens from human intestinal mucosa were obtained from healthy intestinal resections from carcinoma patients undergoing large- or small-bowel surgery. Written consent was obtained before specimen collection, and studies were approved by the local ethics committee. Human intestinal macrophages were isolated from surgical specimens as previously described.[30] Briefly, the tissue was incubated in Hank's balanced salt solution with dithiothreitol (10 mmol/L) for 30 minutes, followed by EDTA (1 mmol/L) treatment for 10 minutes at 37°C to remove intestinal epithelial cells. The tissue was digested in 2 mL phosphate-buffered saline with 1 mg/mL collagenase type I (336 U/mL; Sigma-Aldrich, Munich, Germany), 0.3 mg/mL DNase (Roche, Basel, Switzerland), and 0.2 mg/mL hyaluronidase (2 mg/mL; Sigma-Aldrich) without fetal calf serum (FCS) for 60 minutes at 37°C. After centrifugation through Ficoll–Paque (Sigma-Aldrich), the cells collected from the interface fraction were labeled with CD33 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and sorted by magnetic separation according to the manufacturer's instructions (AutoMACS; Miltenyi Biotec). Cell purity was assessed by flow cytometry (>95% purity was obtained).

Cell Culture and Hypoxia Treatment

The human monocytic cell line MonoMac 6 (MM6; obtained from DSMZ [Leibniz, Germany]) was cultured in RPMI (Sigma-Aldrich, Munich, Germany) supplemented with 10% FCS, 1% nonessential amino acids, and 1% oxalacetic acid–pyruvate–insulin medium supplement (Sigma-Aldrich), and maintained according to American Type Culture Collection recommendations. The human monocytic cell line THP1 was maintained in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (VWR, Dietlikon, Switzerland). The human colorectal adenocarcinoma cell line showing IEC morphology, HT29, was obtained from the German Collection of Cells and Microorganisms (DSMZ) and cultured under conditions as recommended by the DSMZ.

Cells were exposed to hypoxia (0.2% or 2% O_2) in a hypoxia workstation incubator (In vivo 400; Ruskin Technology, Leeds, UK); the addition of cytokines or inhibitors was performed in the hypoxic chamber. Cells that were maintained in normoxia (21% O_2) for the same time period of treatment were used as controls.

pH experiments

The pH shift experiments were performed in serum-free RPMI medium with 2 mmol/L cell culture reagent and 20 mmol/L HEPES. The pH of all solutions was adjusted using a calibrated pH meter (Metrohm, Herisau, Switzerland), the appropriate quantities of NaOH or HCl were added and the medium was allowed to equilibrate in the 5% CO_2 incubator for at least 36 hours. All data presented in this article are referenced to pH measured at room temperature.

Animal Models

All animal experiments were performed according to Swiss animal welfare laws and were approved by the Veterinary Office of the Canton Zurich (Switzerland).

Murine tumor model. C57BL/6/129 mice (age, 6–8 wk) were injected intraperitoneally with a single dose (7.4 mg/kg) of the mutagenic agent azoxymethane followed by 3 cycles of 3% dextran sodium sulfate in drinking water for 1 week and regular drinking water for 2 weeks ad libitum. Mice were killed 10 days after the last cycle for sample collection.

OGR1-deficient mouse model. *Ogr1*$^{-/-}$ (C57BL/6) mice were generated by and obtained from Deltagen, Inc (San Mateo, CA), as previously described.[31]

Genomic DNA Extraction and Genotyping

For PCR reactions, the oligonucleotides used were as follows: *Ogr1* genotyping: 5'-ACCACCAGTGATGCCTAGATCCTGA-3' (P416), 5'-AAGATGACCACGGTGCTGAGCACCA-3' (P417), and 5'-CCATTCGACCACCAAGCGAAACATC-3' (R3).

Murine Macrophage Isolation and Culture

Mature quiescent macrophages from age-matched male *Ogr1*$^{-/-}$ and wild-type (WT) mice (C57BL/6) were isolated from the mouse peritoneal cavity, as previously described.[22] Animals were killed by cervical dislocation to reduce influence of pH. Peritoneal murine macrophages were centrifuged, washed in phosphate-buffered saline, and resuspended in RPMI 1640 medium containing 2 mmol/L Glutamax, 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 2 hours, nonadherent cells were removed and the macrophages were cultured under normoxia or hypoxia at various pH conditions for 24 hours.

RNA Extraction and RT-qPCR

Tissue used for RNA analysis was transferred immediately into RNAlater solution (Qiagen, Valencia, CA) and stored at -80°C. Tissue biopsy specimens were disrupted in RLT buffer (Qiagen) using a 26G needle. Total RNA was isolated using the RNeasy Mini Kit in the automated QIAcube following the manufacturer's recommendations (Qiagen, Hombrechtikon, Switzerland). For removal of residual DNA, DNase treatment, 15 minutes at room temperature, was integrated into the QIAcube program according to the manufacturer's instructions. For complementary DNA synthesis, the High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), was used, following the manufacturer's instructions. Determination of mRNA expression was performed by RT-qPCR on a 7900HT real-time PCR system (Applied Biosystems), under the following cycling conditions: 20 seconds at 95°C, then 45 cycles of 95°C for 3 seconds, and 60°C for 30 seconds with the TaqMan Fast Universal Mastermix (Thermo Fisher Scientific, Wohlen, Switzerland). Samples were analyzed as triplicates. Relative mRNA expression was determined by the comparative ΔΔCt method,[32] which calculates the quantity of the target sequences relative to the endogenous control and a reference sample. TaqMan Gene Expression Assays (all from Applied Biosystems) used in this study were mouse Mm00558545_s1 GPR68 (OGR1), Mm00433695_m1 GPR65 (TDAG8), Mm00446190_m1 IL6, Mm00486332_m1 secreted protein, acidic, cysteine-rich (SPARC), Mm99999068_m1 TNF, mouse β-actin (ACTB) VIC TAMRA (4352341E), human Hs 00268858_s1 GPR68 (OGR1), Hs 00269247_s1 GPR65 (TDAG8), Hs 00270999_s1 GPR4, Hs00174131_m1 IL6, Hs00174103_m1 IL8, Hs00234160_m1 human SPARC, Hs00174128_m1 TNF, and human β-actin Vic TAMRA (4310881E).

Immunoblotting

Total protein was extracted from colon biopsy specimens by lysing homogenized tissue in a RIPA buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP-40) supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany). For each group, a total of 25 μg protein was transferred to a nitrocellulose membrane after electrophoretic separation. The membrane then was incubated overnight with OGR1 primary antibody (AbMart). After washing in Tris-buffered saline, the anti-mouse secondary antibody conjugated to horseradish peroxidase was added, and the membrane was incubated at room temperature for 1 hour. After washing in Tris-buffered saline, the proteins were visualized using the ECL Plus detection kit (Amersham, Velizy-Villacoublay, France). β-actin was used as an internal reference control.

Chromatin Immunoprecipitation Analysis

After the treatment of THP1 cells, ChIP analysis was performed using the ChIP-IT Express Enzymatic kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's instructions. Briefly, after fixation and lysis of the cells, the chromatin was sheared using the enzymatic shearing cocktail. Immunoprecipitation of 25 μg chromatin was performed overnight at 4°C using 3 μg of anti–HIF-1α (BD Biosciences, San Jose, CA) or 3 μg of anti–NF-κB (Cell Signaling, ZA Leiden, The Netherlands) antibodies. After washing the magnetic beads, DNA cross-links of the immune complexes were reverted by heating for 15 minutes at 95°C followed by proteinase K digestion for 1 hour at 37°C. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA and immunoprecipitated DNA using the following promoter-specific primers: for -1680 HIF-1 binding site: 5'-TTGCGTGGCTACTGATTTGC-3' (forward) and 5'-GAACAGTCCAGGAGTAGCCC-3' (reverse), for -1225 NF-κB binding site: 5'-GGGGAAATGCAGTGAATGAGC-3' (forward) and 5'-CAGTGCCAGTGATGTTTGCAT-3' (reverse), for -959 NF-κB binding site: 5'- GATTTGAACCTAGG-CAGTGGG-3' (forward) and 5'- TTTCCAGCCTTAACTGCCTG-3' (reverse), and for -688 HIF-1α and -221 NF-κB binding sites: 5'-GAGCTGCAACACCGCACTTC-3' (forward), 5'-GAACG-CAGGGCCAAGTTGTG-3' (reverse). The PCR products (10 μL) were subjected to electrophoresis on a 2% agarose gel.

Data Analysis and Statistics

Data are presented as means ± SEM for a series of n experiments. Statistical analysis of mouse data was performed using a paired Student *t* test, and probabilities (*P* value, 2 tailed) with a *P* value less than .05 were considered statistically significant. For statistical analysis of groups, 1-way analysis of variance was performed followed by the Tukey post hoc test. Differences were considered significant at a *P* value of less than .05, highly significant at a *P* value of $< .01$, and very highly significant at a *P* value of less than .001.

All authors had access to the study data and reviewed and approved the final manuscript.

Results

OGR1 Expression in Murine Tumor Tissue Is Increased Compared With Normal Tissue

We examined OGR1 mRNA expression levels in a murine colorectal cancer model. OGR1 expression was significantly higher (2.8-fold; $P < .05$) in tumor tissue compared with normal colon tissue. Groups were as follows: control, n = 7; tumor group: n = 7 (Figure 1*A*).

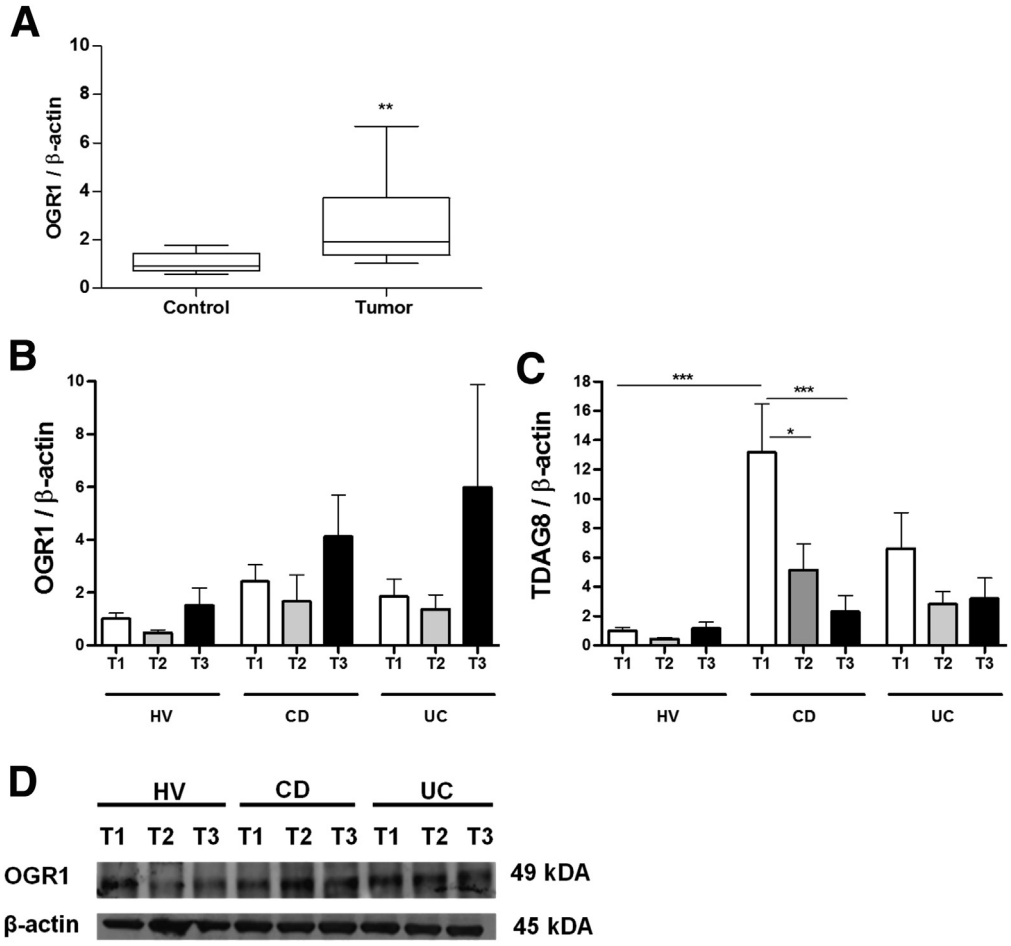


Figure 1. OGR1 expression increases in tumor and under hypoxia. (*A*) OGR1 expression increases in murine tumors. To induce colon carcinomas, mice were injected intraperitoneally with a single dose of azoxymethane (7.4 mg/kg), followed by 3 cycles of 3% dextran sodium sulfate in drinking water for 1 week and normal drinking water for 2 weeks. Mice were killed 10 days after the last cycle for colonic tumor collection. OGR1 mRNA expression levels were significantly higher ($P = .0220$) in murine colonic tumors compared with the normal colonic mucosa. Groups: control (n = 7), tumor group (n = 7). (*B* and *C*) Hypoxia induces a tendency to increase OGR1 mRNA expression and significantly decreases TDAG8 mRNA expression in the colon of IBD patients. HVs (n = 10), CD patients (n = 11), and UC patients (n = 9) were subjected to hypoxic conditions in a hypobaric chamber resembling an altitude of 4000 m for 3 hours. Distal colon biopsy specimens were taken the day before entering the hypobaric chamber (T1), immediately after hypoxia (T2), and 1 week after the first biopsy (T3). Total RNA was isolated, reverse-transcribed, and hypoxia-induced changes in gene expression were analyzed using RT-qPCR. (*B*) Although not significant, OGR1 mRNA expression after hypoxia showed an increasing tendency at T3 in CD and UC patients when compared with HVs. (*C*) Conversely, mRNA levels of TDAG8 were reduced significantly at T1 and T3 in CD patients subjected to hypoxia. Expression changes were calculated relative to samples taken at T1 after normalizing with human β-actin endogenous control. (*D*) Immunoblotting analysis of OGR1 in colon biopsy specimens from HVs, CD patients, and UC patients as described earlier. β-actin was used as loading control. Results represent means ± SEM. Statistical analysis was performed using 1-way analysis of variance followed by the Tukey test (*$P < .05$, **$P < .01$, ***$P < .001$).

IBD Patients Under Hypoxia Show an Enhanced OGR1 mRNA Expression When Compared With Healthy Controls

To study the effects of hypoxia on the expression of the pH-sensing receptors OGR1 and TDAG8 in the colon of human subjects, HVs (n = 10), CD patients (n = 11), and UC patients (n = 9) were subjected to hypoxic conditions in a hypobaric chamber resembling an altitude of 4000 m above sea level for 3 hours. Although not significant, the mRNA expression of OGR1 showed a clear trend to an increase 1 week after hypoxia in CD and UC patients when compared with HVs (Figure 1*B*). In addition, Western blot analysis of OGR1 showed that the expression of OGR1 was increased in the colon of CD and UC patients after exposure to hypoxia as compared with the control group (Figure 1*D*). Conversely, mRNA levels of TDAG8 in CD patients, but not HVs, were reduced significantly at T2 and T3 after hypoxia when compared with T1, and a similar trend was shown in UC patients (Figure 1*C*).

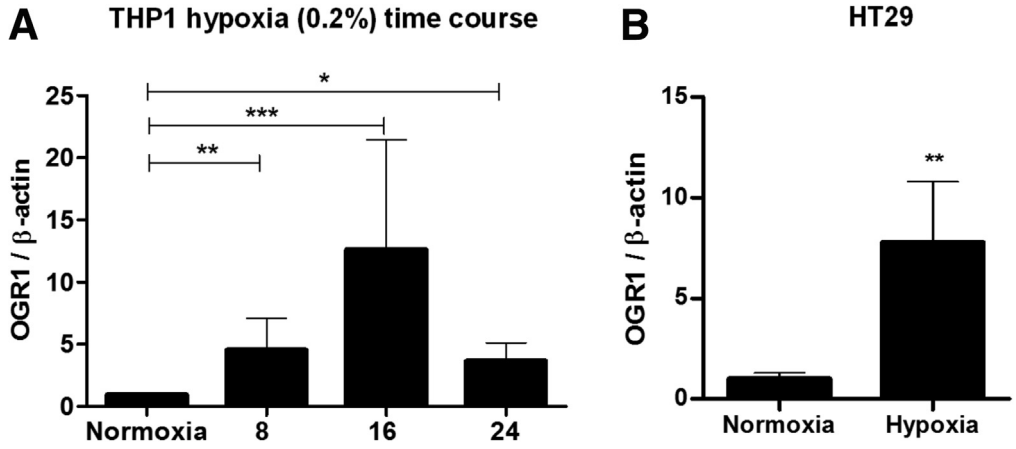


Figure 2. Hypoxia induces the mRNA expression of OGR1 in cultured human IECs and monocytes. (*A* and *B*) HT29 and THP1 cells were incubated under hypoxic conditions (0.2% O_2) for 8, 16, and 24 hours. Total RNA was isolated, reverse-transcribed, and hypoxia-induced changes in gene expression were analyzed using RT-qPCR. Hypoxia significantly induced the mRNA expression of OGR1. Results represent means ± SEM of 2 independent experiments. Statistical analysis was performed using the Student *t* test (n = 5; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$).

Hypoxia Induces OGR1 Expression in Cultured Human IECs and Monocytes

Because our results suggest a positive regulation of OGR1 expression in IBD patients under hypoxia, we sought to confirm this effect in IECs and monocytic cells because these cell types cells play a major role in gut innate immunity and homeostasis. For this purpose, we subjected cultured human monocytic THP1 cells to hypoxia (0.2% O_2) for 8, 16, and 24 hours, and the IEC line HT29 for 24 hours. Hypoxia significantly induced OGR1 mRNA expression in THP1 cells

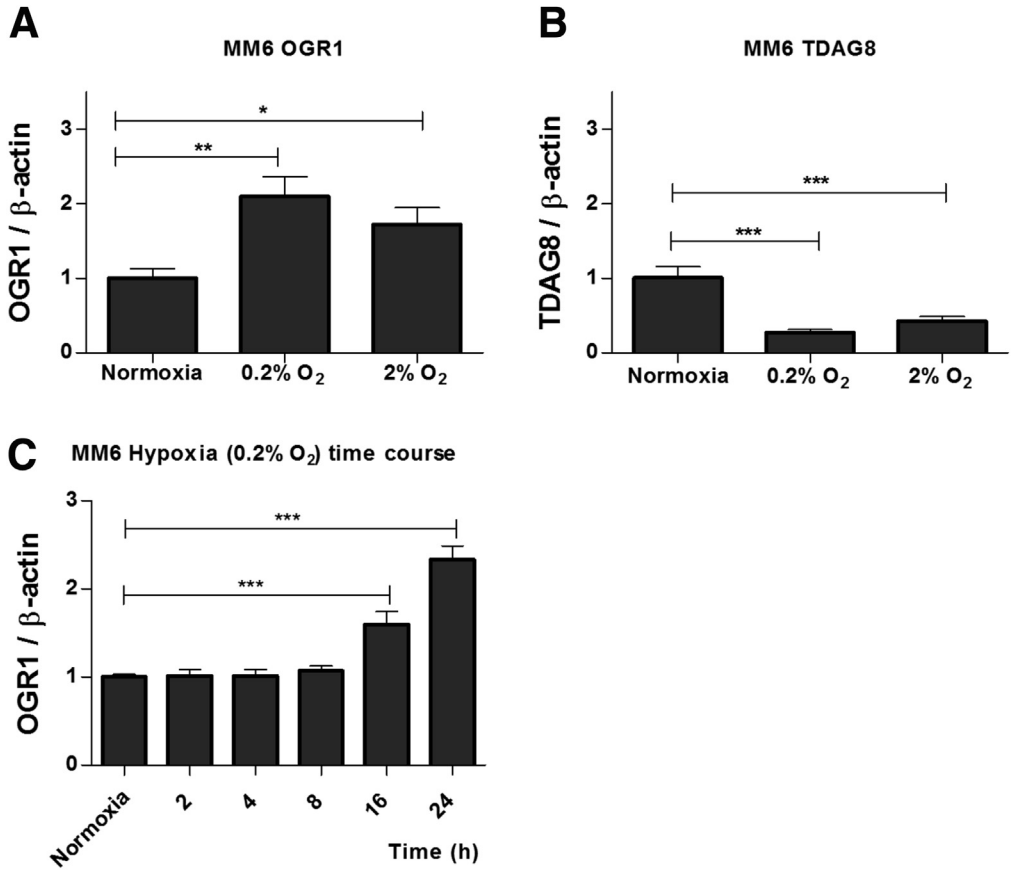


Figure 3. Expression of pH-sensing receptors OGR1 and TDAG8 changed under hypoxic conditions. MonoMac 6 cells were exposed to quite severe hypoxia (0.2% O_2) or modest hypoxia (2% O_2) for 18 hours. (*A*) OGR1 expression increased 2-fold and 1.7-fold at 0.2% and 2% O_2, respectively. (*B*) Conversely, TDAG8 mRNA expression decreased 0.7- and 0.6-fold at 0.2% and 2% O_2, respectively. (*C*) OGR1 mRNA expression remained unchanged from 0 to 8 hours, but increased 1.6- and 2.3-fold after 16 and 24 hours, respectively. Each figure is representative of at least 3 independent experiments. Results are expressed as means ± SD. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ using 1-way analysis of variance followed by the Tukey test.

(4.76-, 13.19-, and 3.82-fold increase; $P = .0067$, $P = .0005$, $P = .02$) at 8, 16, and 24 hours, respectively (Figure 2*A*), and HT29 cells (7.78-fold increase; $P = .0042$) (Figure 2*B*).

We next investigated the effects of hypoxia on the expression of the pH-sensing receptors in the human cell line MM6, which shows characteristics and functional features of mature blood monocytes.[33,34] Expression levels of the pH-sensing receptors OGR1, GPR4, and TDAG8, in MM6 cells exposed to severe hypoxia (0.2% O_2) or modest hypoxia (2% O_2) for 18 hours, were examined by RT-qPCR. OGR1 mRNA expression increased (2- and 1.7-fold; $P < .05$ and $P < .01$) at 0.2% and 2% O_2, respectively (Figure 3*A*). Conversely, TDAG8 expression decreased (0.7- and 0.6-fold; $P < .001$ and $P < .001$) at 0.2% and 2% O_2, respectively (Figure 3*B*). The expression of GPR4 in MM6 cells was very low and hypoxia did not significantly affect GPR4 gene expression (data not shown). Because GPR4 is only weakly expressed in myeloid cells and IECs, further experiments in this study focused only on OGR1 and TDAG8.

To determine the induction time required to induce OGR1 expression in MM6 cells under hypoxia (0.2% O_2), we selected 5 different time points (0–24 h); no change was detected at 8 hours, but at time points of 16 and 24 hours expression increased 1.6- and 2.3-fold, respectively ($P < .001$ and $P < .001$) (Figure 3*C*).

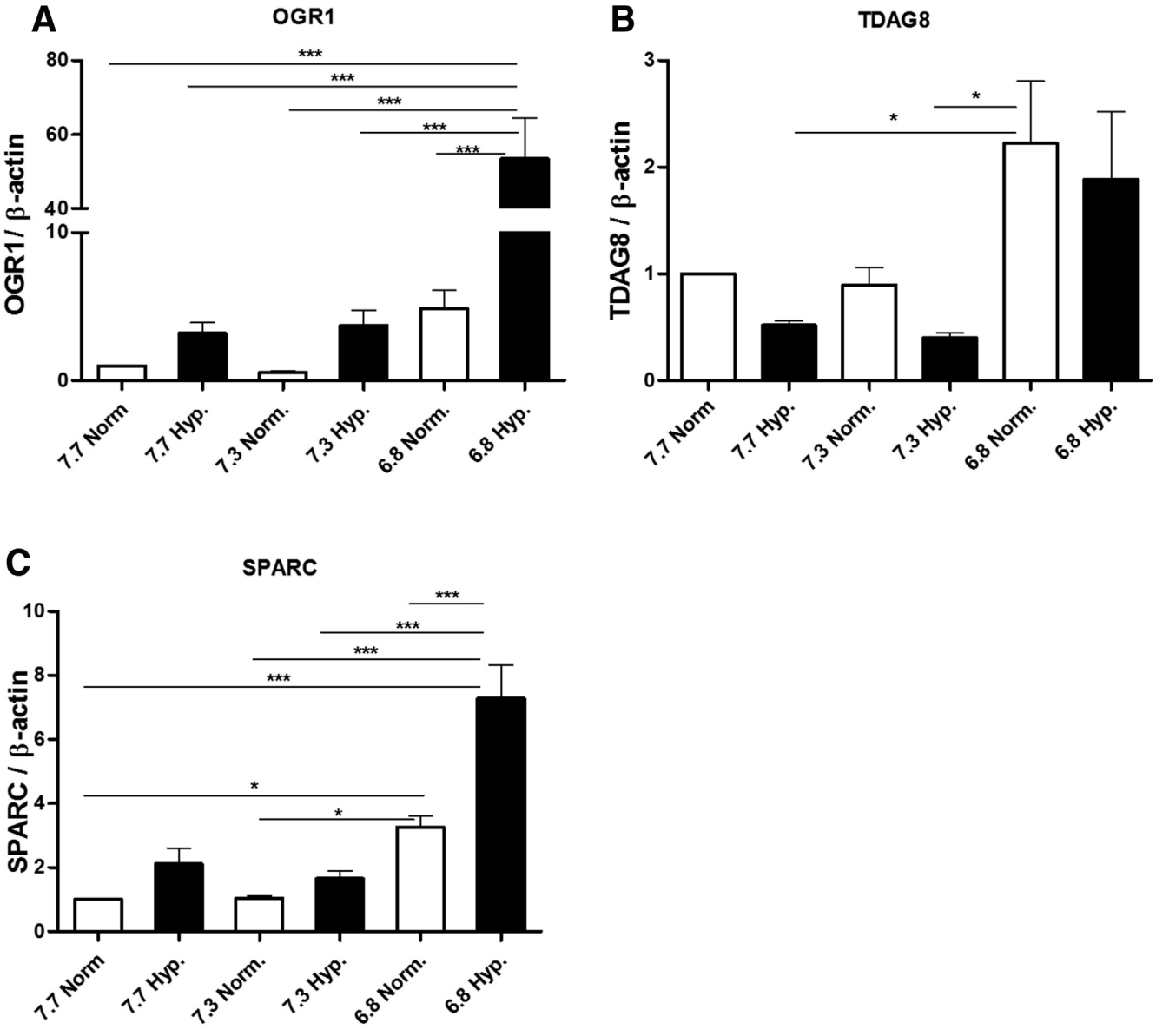
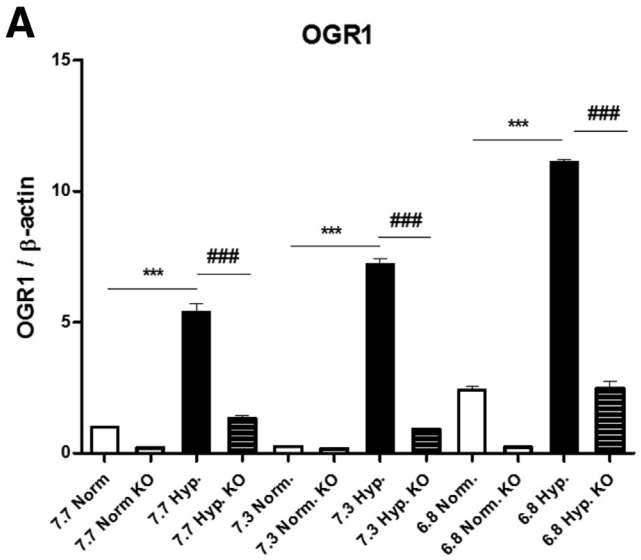


Figure 4. Expression of pH-sensing receptors OGR1 and TDAG8 under hypoxic conditions was influenced further by pH in MM6 cells. MonoMac 6 cells were exposed to hypoxia (0.2% O_2) for 24 hours at pH 7.7, pH 7.3, and pH 6.8. Under hypoxic conditions: (*A*) OGR1 mRNA expression increased at acidic pH compared with expression at pH 7.7 or pH 7.3, (*B*) TDAG8 mRNA expression decreased in all conditions under hypoxia (ns), and (*C*) SPARC mRNA expression was increased significantly under hypoxia at pH 6.8. Results represent means ± SEM of 5 independent experiments. Asterisks denote significant differences from the respective control: $^{*}P < .05$, and $^{***}P < .001$.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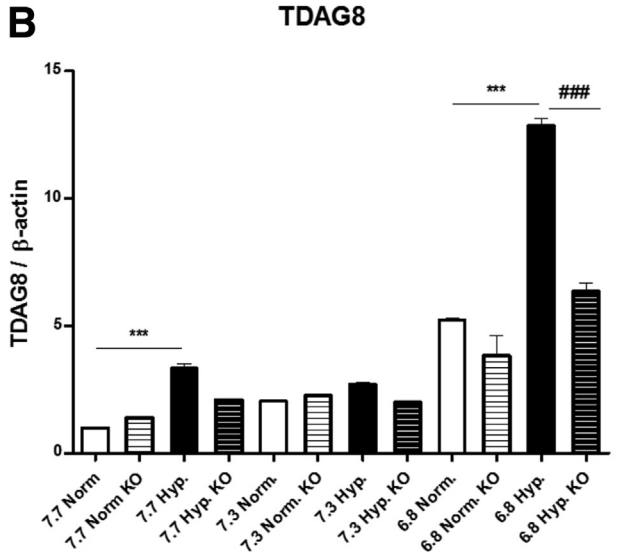
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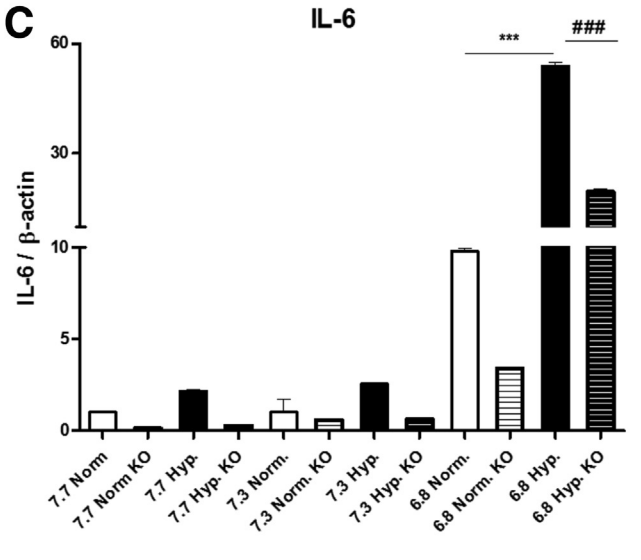
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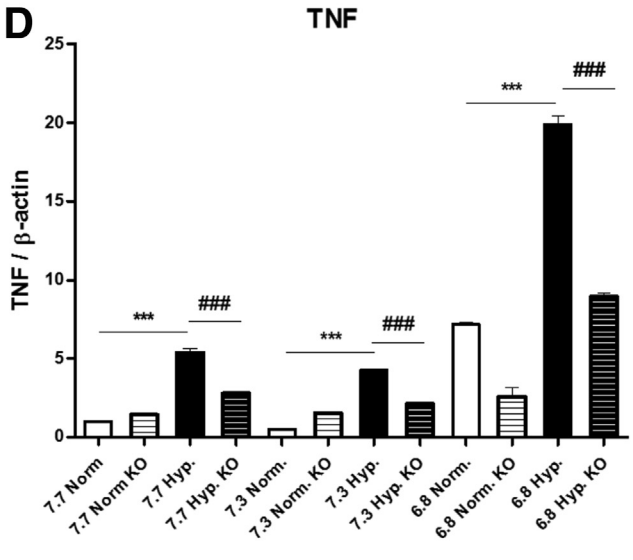
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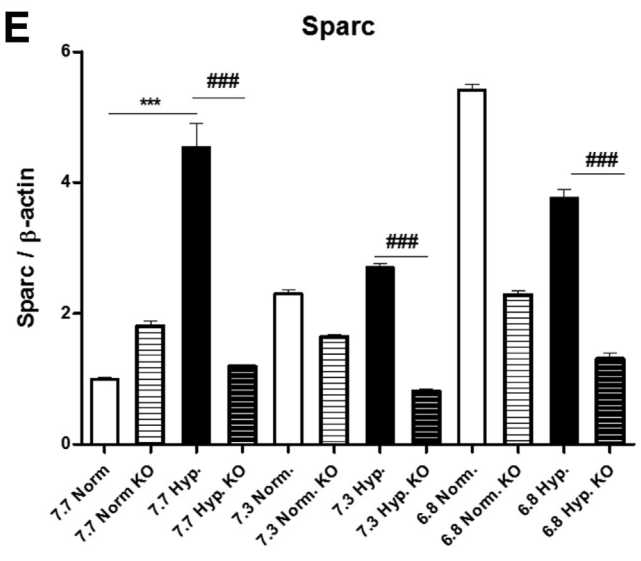
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Acidic pH Induces OGR1 and OGR1-Dependent mRNA Expression in MM6 Cells Subjected to Hypoxia

To assess the influence of pH in MM6 cells under hypoxia, we compared expression levels of OGR1 and OGR1-dependent genes at pH 7.7, 7.3, and 6.8 at hypoxic conditions (0.2% O_2 for 24 h) to normoxia, pH 7.7. Hypoxia induced a higher expression of OGR1 in all pH levels analyzed, with the highest increase at the acidic pH. OGR1 expression levels of MM6 cells under hypoxia increased 3.2-, 3.7-, and 53.5-fold ($P < .001$, $P < .001$, and $P < .001$) at pH 7.7, 7.3, and 6.8, respectively, compared with pH 7.7, normoxia (Figure 4*A*). Under hypoxia, OGR1 expression at acidic pH increased more than 14-fold compared with pH 7.7 and pH 7.3. Conversely, MM6 cells exposed to hypoxia at pH 7.7, 7.3, and 6.8 showed a trend toward a decreased TDAG8 expression compared with the respective normoxic conditions (Figure 4*B*).

In an earlier study, we observed that OGR1-induced gene expression in response to extracellular acidification in murine macrophages was enriched for inflammation and immune response, actin cytoskeleton, and cell-adhesion gene pathways.[22] Therefore, in this study we examined the effect of hypoxia on the expression levels of the highly regulated OGR1-dependent gene SPARC, an extracellular matrix–associated protein known to regulate the expression of matrix metalloproteinase and cytoskeleton architecture.[35,36] An acidic pH of 6.8 resulted in a 3.2-fold ($P < .001$) induction of SPARC expression at normoxia and 7.3-fold ($P < .001$) at hypoxia compared with pH 7.7 normoxia (Figure 4*C*).

Acidic pH Induces the Expression of pH Receptors and Proinflammatory Cytokines in Murine Peritoneal and Human Intestinal Macrophages Under Hypoxia

To confirm the effect of hypoxia and acidic pH on the expression of pH receptors and proinflammatory cytokines in primary macrophages, we isolated peritoneal macrophages from *Ogr1*$^{-/-}$ and WT mice and intestinal macrophages from healthy intestinal tissue from colon carcinoma patients. The expression of OGR1, but not TDAG8, was increased significantly under hypoxia at pH 7.7, 7.3, and 6.8, with a higher increase of OGR1 expression at acidic conditions (Figures 5 and 6*A* and *B*). This result suggests that the expression of OGR1 and TDAG8 under hypoxia is regulated differentially at acidic conditions. Interestingly, hypoxia also induced the expression of the proinflammatory cytokines IL6, IL8, and TNF, but not SPARC, with a significantly higher expression at acidic conditions (Figures 5 and 6*C* and *F*). Importantly, proinflammatory gene expression was reduced significantly in macrophages from OGR1$^{-/-}$ mice compared with WT mice, confirming a crucial role for OGR1 in hypoxia-mediated changes in these genes.

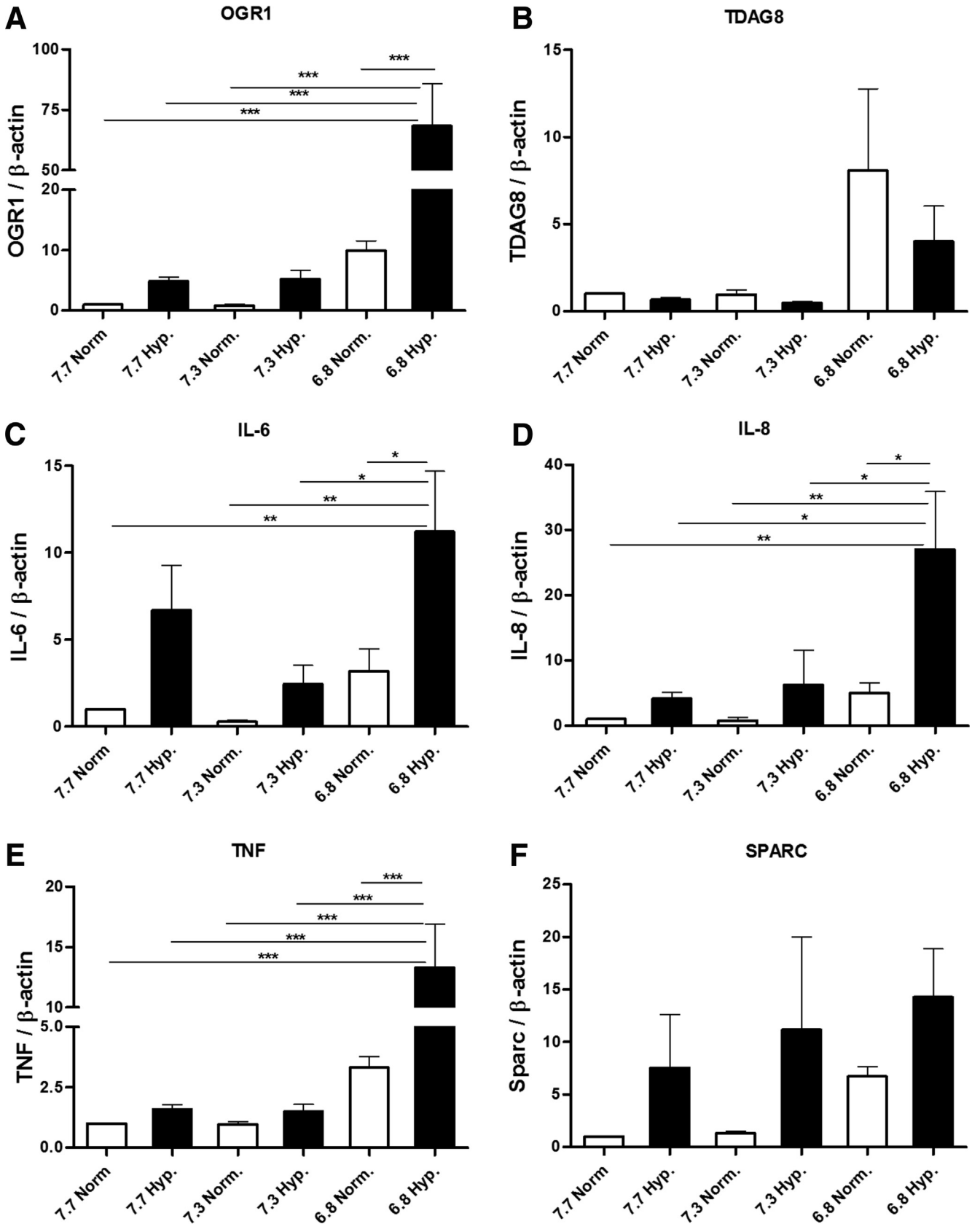
TNF-Induced OGR1 Expression Is Enhanced Under Hypoxia and Reversed in the Presence of NF-κB Inhibitors

In a previous study we observed that treatment of MM6 cells with TNF led to a significant up-regulation of OGR1 expression through a NF-κB–mediated mechanism, with the maximum OGR1 expression occurring between 6 and 8 hours.[22] This prompted us to examine the additional influence of hypoxia on TNF-treated MM6 cells in the presence or absence of NF-κB inhibitors. Our results show that control/nontreated cells exposed to hypoxia for 6 and 24 hours increased 1.13- and 9.8-fold ($P =$ not significant and $P < .001$), respectively (Figure 7*A* and *B*). Furthermore, OGR1 expression levels in MM6 cells treated with TNF (25 ng/mL) and exposed to hypoxia (0.2% O_2) for 6 and 24 hours, increased 1.5- and 17.8-fold ($P < .0001$, .001) respectively, compared with the corresponding treatment at normoxic conditions (Figure 7*A* and *B*). Interestingly, hypoxic conditions and TNF stimulation showed a synergistic effect in the induction of OGR1 expression at 24 hours (Figure 7*B*).

Cells treated with TNF in the presence of the NF-κB inhibitor SC-514 (25 μmol/L), which is known to inhibit the kinase activity of inhibitor of kappa B kinase,[37] showed decreased OGR1 expression at normoxia (3.9-fold or 68% decrease; $P < .001$) and hypoxia (5.74-fold or 80% decrease; $P < .001$), relative to the corresponding controls. The degree of inhibition between normoxic and hypoxic conditions showed no significant difference. However, the inhibitory effect of SC-514 was less effective on longer exposure to hypoxia (24 h) and we observed a 9.5-fold or 42% decrease ($P < .001$) in OGR1 expression compared with normoxia (3.26-fold or 78% decrease; $P < .001$) (Figure 7*B*).

In addition to SC-514, we also tested AICAR ribonucleoside, which blocks the expression of proinflammatory cytokines (TNF, IL1β, and IL6), inducible nitric oxide synthase, prostaglandin-endoperoxide synthase 2, and manganese superoxide dismutase mRNAs in glial cells and macrophages by inhibiting NF-κB and CCAAT/enhancer binding protein beta pathways.[38] Nontreated or controls cells exposed to hypoxic conditions induced a 1.83-fold increase ($P < .05$) in OGR1 expression compared with cells at normoxic conditions (Figure 7*C*). TNF-treated cells in the presence of 0.05

Figure 5. (***See previous page***). **Expression of pH-sensing receptors OGR1 and TDAG8 under hypoxic conditions was influenced by pH in peritoneal macrophages from WT mice but not from *Ogr1*$^{-/-}$ mice.** Mature quiescent peritoneal macrophages from *Ogr1*$^{-/-}$ and WT mice were isolated and cultured at different pH values (7.7, 7.3, and 6.8) under normoxia or hypoxia (0.2% O_2) for 24 hours. mRNA isolation was performed and the expression of several genes was measured by RT-qPCR. (*A*) OGR1 mRNA expression was significantly higher in hypoxia at acidic pH. (*B*) TDAG8 mRNA expression was significantly higher in hypoxia at acidic pH values in WT cells. (*C* and *D*) The mRNA expression of proinflammatory cytokines, IL6, and TNF was significantly higher in hypoxia at pH 6.8, with an approximately 2-fold increase in WT cells. (*E*) At acidic pH under hypoxia expression of SPARC was not increased. Results are expressed as means ± SEM (n = 3). ***$P < .001$ using 1-way analysis of variance followed by the Tukey test.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mmol/L AICAR showed a 41% decrease (1.2-fold decrease; $P < .001$) compared with cells in the absence of the inhibitor (Figure 7*C*). The same treatment under hypoxic conditions resulted in a comparable decrease (35% or 1.5-fold decrease; $P < .001$). In the presence of SC-514 together with TNF, OGR1 expression decreased 59% (1.7-fold decrease; $P < .001$) compared with the relevant control without SC-514. However, under hypoxic conditions the degree of inhibition decreased significantly (40% decrease equivalent to a 1.6-fold decrease; $P < .001$) (Figure 7*C*).

Transcriptional Regulation of OGR1 Under Hypoxia Is Mediated by HIF-1α in THP1 Cells

After our results on the induction of OGR1 expression under hypoxic conditions, we sought to elucidate the molecular mechanisms governing transcriptional regulation of OGR1 under hypoxia. Sequence analysis of the OGR1 promoter identified several putative binding sites for the transcription factors NF-κB and HIF-1α. To investigate the binding activity of HIF-1α and NF-κB to a promoter of OGR1, we performed ChIP analysis of promoter regions containing HIF-1α and NF-κB binding sites in THP1 cells after hypoxia (0.2 % O_2, 24 h) (Figure 8*A*). ChIP analysis showed that HIF-1α, but not NF-κB, binds to the promoter of OGR1, 24 hours after hypoxia in THP1 cells (Figure 8*B*).

Discussion

Tissue hypoxia stimulates multiple responses, including glycolysis and the extrusion of lactic acid and protons, thereby decreasing extracellular pH.[39–41] One of the mechanisms responsible for the high rate of glycolysis is HIF-1α, the main hypoxic switch,[42] which up-regulates the expression of the glucose transporters GLUT1 and GLUT3,[43] and the activation of the glycolytic enzymes lactate dehydrogenase,[44] pyruvate dehydrogenase kinase-1,[45] and pyruvate dehydrogenase kinase-3.[46] Tumor environment is characterized by hypoxia and low pH, particularly at the core. Together with GRP4, OGR1 and TDAG8 are major proton-sensing G-protein–coupled receptors that have been shown to play a role in cancer, immune cell function, and inflammation.[7,22,47,48] In this study we showed that the mRNA expression of the pH sensor OGR1 was increased 2-fold in murine tumor tissue. Although small, the induction of OGR1 in tumor tissue is in good agreement with the known interaction between tumor hypoxia and acidosis,[49] and several putative DNA binding sites for HIF-1α within the proximal regions of the OGR1 promoter variants have been identified.[22]

In accordance with the severe hypoxic environment and increased acidosis observed in the intestinal mucosa in IBD,[50,51] we also show that OGR1 expression is up-regulated approximately 2-fold in CD and UC patients, however, 1 week after hypoxia we observed a 4- and 6-fold increase in OGR1 expression in CD and UC patients, respectively, when compared with healthy subjects. This result indicates that OGR1 is an important regulatory factor contributing to the onset of mucosal inflammation and a marker for IBD. Interestingly, hypoxia caused by high altitude or airplane flights has been shown to trigger flares of human IBD,[52] which may be mediated partially by changed expression of transporter proteins and cytokine signaling.[53] Interestingly, the constitutive expression of TDAG8 was markedly higher in CD and UC patients compared with healthy subjects. Conversely to OGR1, the expression of TDAG8 was reduced significantly in CD patients immediately and 1 week after hypoxia, and showed a trend toward a reduced expression in UC patients subjected to hypoxia. This down-regulation suggests a role for TDAG8 in the negative regulation of proinflammatory responses, and would be in agreement with several reports showing the involvement of TDAG8 in the inhibition of immune responses.[54,55] Interestingly, a recent report showed that TDAG8 contributes to the inhibition of cytokine production from extracellular acidification in peritoneal macrophages.[7] Taken together, these results point to a different function of the 2 pH sensors OGR1 and TDAG8 in the regulation of inflammatory responses. Reduction of TDAG8 expression under hypoxia would reduce inhibitory effects on proinflammatory gene expression, whereas increased OGR1 expression would mediate proinflammatory responses directly.

We also show that OGR1 expression increases in human monocytic cell lines, IECs, and macrophages subjected to hypoxia, suggesting a pivotal role of OGR1 in hypoxia-induced responses. In a previous study using $OGR1^{-/-}$ mice, we showed that OGR1 plays a crucial role in the expression of several main factors involved in immune responses and inflammation, including IL6, TNF, IL8, and SPARC,[22] a master regulator of tissue remodeling and epithelial-mesenchymal transition (EMT).[35,36] Accordingly, the expression of IL6, TNF, IL8, and SPARC was increased concomitantly with OGR1 under hypoxic and acidic conditions. Interestingly, the expression of IL6 and TNF was reduced significantly in macrophages from $OGR1^{-/-}$ mice, confirming an essential role for OGR1 in hypoxia-mediated inflammatory responses. Taken together, these results suggest that OGR1 may play a crucial role in inflammation and metabolic homeostasis under hypoxia and acidic extracellular pH. In agreement with this, previous studies have shown a critical role for OGR1 in proinflammatory cytokine expression and tissue remodeling after extracellular acidification.[56,57]

Figure 6. (***See previous page***). **Expression of pH-sensing receptors OGR1 and TDAG8 under hypoxic conditions was influenced further by pH in human intestinal macrophages.** Intestinal macrophages from healthy mucosa of carcinoma patients were isolated and cultured at different pH values (7.7, 7.3, and 6.8) under normoxia or hypoxia (0.2% O_2) for 24 hours. mRNA isolation was performed and the expression of several genes was measured by RT-qPCR. (*A*) OGR1 mRNA expression was significantly higher in hypoxia at acidic pH. (*B*) TDAG8 mRNA expression was reduced in hypoxia in all pH levels tested. (*C–E*) The mRNA expression of proinflammatory cytokines, IL6, IL8, and TNF was significantly higher in hypoxia at pH 6.8. (*F*) No differences in the mRNA expression of SPARC. Results are expressed as means ± SEM (n = 5). $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ using 1-way analysis of variance followed by the Tukey test.

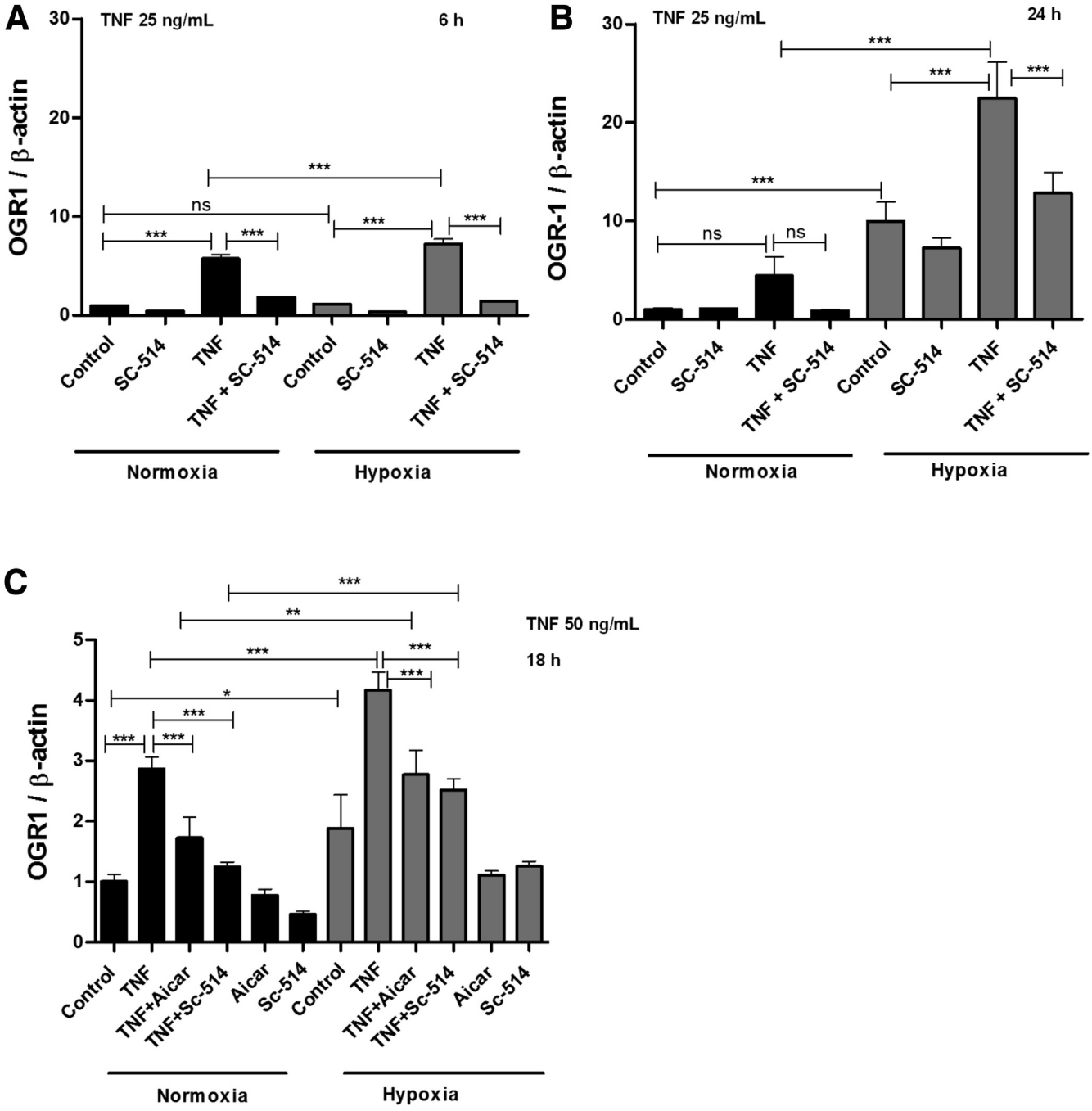

Figure 7. Hypoxia enhances TNF induction of OGR1 expression and was reversed by NF-κB inhibitors in MM6 cells. TNF treatment under hypoxia (0.2% O_2). (*A* and *B*) After 6 hours, OGR1 mRNA expression was enhanced, but after 24 hours there was a synergistic effect by hypoxia on TNF induction. The inhibitory effect of the NF-κB inhibitor SC-514 (25 μmol/L) was less effective under hypoxia. (*C*) AICAR (0.05 mmol/L) showed less inhibitory effects under hypoxia. Each figure is representative of 3 independent experiments. Results are expressed as means ± SD. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ using 1-way analysis of variance followed by the Tukey test.

We show that hypoxia enhances TNF-mediated induction of OGR1 expression. In a previous study we reported that TNF treatment induced OGR1 expression in MM6 cells and primary human and murine monocytes, and that this process was reversed by the NF-κB inhibitors MG132, AICAR, BAY-11-7082, CAY10512, and SC-514 in MM6 cells.[22] In the current study we show that hypoxia enhances TNF-mediated induction of OGR1 expression, and that this effect was reversed by NF-κB inhibition under hypoxic conditions. The 2 central transcription factors, HIF-1α and NF-κB, involved in the regulation of decreased oxygen availability are known to show an intimate interdependence at several mechanistic levels.[58] In a previous study we showed that an in silico analysis using MatInspector software (Munich, Germany) and

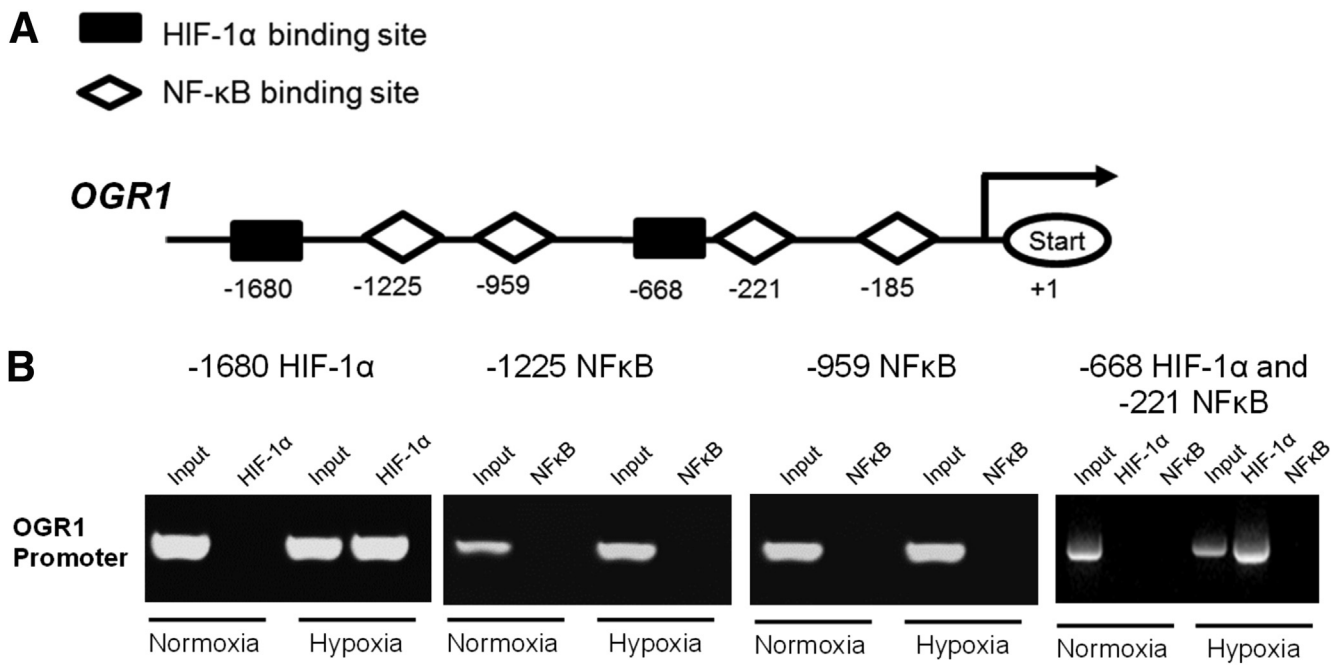

Figure 8. Hypoxia induces the recruitment of HIF-1α, but not NF-κB, to the OGR1 promoter. (*A*) Putative binding sites for HIF-1a and NF-κB were found in the OGR1 promoter using Genomatix software tools (Munich, Germany). (*B*) THP1 cells were incubated under hypoxic conditions (0.2% O_2) for 24 hours. ChIP analysis was performed using antibodies against HIF-1α and NF-kB, for immunoprecipitation. RT-qPCR was performed using the TaqMan system with specific primers for the OGR1 promoter-binding sites of the nuclear factors HIF-1α and NF-κB. Aliquots taken before immunoprecipitation were used as input control. PCR products were run on 2% agarose gel. The results are representative of 2 independent experiments. HIF-1α, but not NF-κB, was recruited to the promoter of OGR1 in THP1 cells subjected to hypoxia for 24 hours.

visual inspection showed several putative DNA binding sites for NF-κB and HIF-1α within the proximal regions of the OGR1 promoter variants.[22] Accordingly, ChIP analysis of promoter regions of OGR1 containing binding sites for HIF-1α and NF-κB showed that hypoxia induced the binding of HIF-1α to the OGR1 promoter in 2 different binding sites, -1680 and -668, confirming that OGR1 is under the transcriptional control of HIF-1α. Of note, hypoxia did not induce the binding of NF-κB to the -221, -959, and -1225 promoter binding sites, suggesting an alternative mechanism for NF-κB to induce OGR1 expression that does not involve binding of NF-κB to this promoter. Interestingly, a recent study using rabbits subjected to hypoxia has shown that TNF induces the binding of HIF-1α to the promoter of HIF-1α target genes through a NF-κB–mediated mechanism that does not require the binding of NF-κB to the promoter.[59] This result could explain the induction of OGR1 expression observed in TNF-treated cells under hypoxia and the reversion of this effect with NF-κB inhibitors. Furthermore, it has been shown that not only hypoxic induction but also an increase in hydrogen ions results in transient and reversible loss of von Hippel-Lindau function by promoting its nuclear sequestration,[49] causing activation of HIF-1α.

Conclusions

In this study we show that the hypoxic environment triggers the expression of the pH-sensing receptor OGR1, and this induction was enhanced at acidic pH, a common feature of tumors and tissue inflammation, suggesting a role for OGR1 in the physiological regulation associated with hypoxia and tissue acidification. We previously reported that OGR1 expression was induced in cells of human macrophage lineage and primary human monocytes by TNF and that NF-κB inhibition reversed the induction of OGR1 mRNA expression by TNF. Here, we report that hypoxia, known to cross-talk with the NF-κB pathway, enhanced the TNF-mediated induction of OGR1 expression, which was reversed in the presence of NF-κB inhibitors. The stimulation of OGR1 expression by TNF and hypoxia, and subsequent pH-sensing activity, may play a role in IBD pathogenesis.

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Conflicts of interest
The authors disclose no conflicts.

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